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Human immunodeficiency virus type-1 (HIV-1) Tat protein is a transactivator of viral gene expression. Tat as an over-expressed fusion protein, Rop-Tat has been purified after cyanogen bromide cleavage by using S-Sepharose Q-Sepharose column chromatography and subsequent use of HPLC. It is about 90% pure and the major impurity is a peptide which is 4 amino acids shorter than Tat, caused by breaking the acid labile bond of aspartate-proline under the acidic cleavage condition of cyanogen bromide. The fusion protein, Rop-Tat was purified by using the same procedures used for Tat. The next step will be refolding of Tat and Rop-Tat.

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Introduction

Human immunodeficiency virus type-1 (HIV-1) transactivator Tat is a transactivator of viral gene expression. The Tat protein has been overexpressed in a prokaryotic expression system developed by Dr. Ru Chih C. Huang (co-principal investigator). This overexpressed system produces a fusion protein, Rop-Tat (1). Rop is a 7.2 kDa protein with 63 amino acids whereas Tat consists of 86 amino acids, including 7 cysteine residues followed by a host of basic amino acids (2,3,4). Together, the fusion protein, Rop-Tat, has a molecular weight of 15.8 kDa. On SDS-gel electrophoresis the Rop-Tat is slightly below the 20 kDa molecular weight standard. The fusion protein, Rop-Tat, is accumulated as insoluble aggregates of inclusion bodies (IB). In our first year of research (May 1993 to May 1994), we prepared the IB from the overexpressed cell cultures of *E coli* and then used cyanogen bromide cleavage at methionine. The fusion point between Rop and Tat is a methionine residue, and since Tat does not have any methionine, it is expected to produce an intact Tat protein after cyanogen bromide treatment. The cyanogen bromide digestion is followed by Q and S cation and anion column chromatography. We isolated and purified from the SDS gel electrophoresis a protein having a molecular weight of about 9.9 kDa, which is very close to Tat (9.7 kDa). Using another low molecular weight gel electrophoresis, this 9.9 kDa protein band appeared under the 8.1 kDa standard. Since only 52 out of 63 amino acids of Rop protein are fused with Tat and since the residue number 11 of Rop protein is a methionine, cyanogen bromide treatment is expected to produce residue 12 to 53 of Rop. This residue has only a molecular weight of about 4.8 kDa, and therefore we did not think the protein we purified was Rop. However, when this protein fraction was subjected to amino acid

sequence analysis, it proved to be the residue 12-53 of the Rop protein. This Rop peptide may exist in the form of a dimer. The mistake we made is that we should have sent this fraction out to be analyzed for amino acid sequencing before we put our entire effort into its purification and therefore wasting a lot of time. In this second year of research we concentrated on first purifying the Tat protein. Then we also purified the Rop-Tat protein.

I. Cyanogen Bromide Cleavage of the Fusion Protein, Rop-Tat, and Purify Tat Protein

II. Purification of the Fusion Protein, Rop-Tat

III. Conclusion

Body of the Report

I. Isolation and Purification of Tat Protein:

The Rop-Tat protein is currently being isolated from IB. The IB preparation was described in our last annual report and need not be repeated here. Since the IB do not dissolve in 70% formic acid (the solvent used for cyanogen bromide digestion), the procedure of Lee and Christie (5) was followed in order to solubilize the Rop-Tat from the IB. This procedure was used for extraction of Ogr protein from IB. Rop-Tat was precipitated into an ammonium sulfate pellet. The pellet was able to dissolve in 70% formic acid, cyanogen bromide was added in the amount of 2 mg per 20-30 mg of protein. It was then incubated at room temperature under nitrogen for 24 hours. The solution was dialyzed against 10 mM HCl extensively. It was then dialyzed against 1 liter of 5 M urea, 50 mM sodium acetate at pH 5 for 2 days. The solution was then passed through a 2.5 x 6 cm S-Sepharose column. The pH of the 0.5 M fraction from S-Sepharose was adjusted to pH 8 by using NaOH. The 0.5 M elute fraction was then dialyzed against 5 M urea, 0.02

M sodium phosphate, 25% acetonitrile at pH 8 for 2 days. An S-Sepharose column (1.5 x 6 cm) was equilibrated with the acetronitrile-containing buffer and eluted with 0.1 M, 0.2 M, 0.33 M, 0.5 M and 1 M NaCl added to the column buffer. The 0.33 M fraction is free of low molecular weight Rop, and is subjected to HPLC to get rid of high molecular weight contaminants. Reverse phase (Vydac C-18 218TP54) was used. The main contaminant is a band close to the Tat band (see Lane 1, Figure 1). Even after HPLC this band is not completely separated from Tat, but comes out as a shoulder peak. The Lane 2, Figure 1 sample was subjected to commercial amino acid analysis and it proved to be about 90 % pure. The major contaminant was a peptide slightly shorter by 4 amino acids than the complete Tat protein due to breakage of the acid labile asp-proline bond of Tat protein. Since the acid condition is necessary for obtaining Tat protein from Rop-Tat, it will be difficult to avoid breaking this acid labile bond. From earlier work we know that Rop-Tat is also functionally active. Similar chromatography procedures can also be used to purify the Rop-Tat, and then the acid condition will be unnecessary and the purity can be increased. We have tried many different approaches to purify the Rop-Tat protein because the solubility of this fusion protein is much less than that of the Tat protein. Three different strategies are used to purify the Rop-Tat protein.

II. Purification of the Fusion Protein, Rop-Tat

Method 1:

IB were first solubilized following the procedure of Christie *et al* (5). The pellet from the ammonium sulfate was washed with 0.05 M tris, 0.05 M EDTA, 5% triton X-100 at pH 7.7, sonicated, centrifuged at 15,000 rpm for 15 min. This process of washing

with triton X-100 buffer was repeated once. The pellet was then washed with the tris buffer without the triton X-100, sonicated and centrifuged twice more. The pellet was dissolved (with the aid of sonication) into 6 M urea, 0.05 M tris at pH 7.6 and applied onto a 2.5 x 12 cm Q-Sepharose column previously equilibrated with the same buffer. After washing with the same buffer, the column was eluted with a gradient of 0.1 M to 0.5 M NaCl added to the urea and tris buffer. The results are shown on Figure 2. Only the run-through peak has Rop-Tat protein in it. The run-through peak was directly applied onto an S-Sepharose (2.5 x 12 cm) column previously equilibrated with 6 M urea, 0.05 M sodium acetate at pH 5. The column was then eluted with 0.1 M NaCl to 0.5 M NaCl in the above acetate and urea buffer. The results are shown on Figure 3. Only Peak 4 of Figure 3 has Rop-Tat. Peaks from both columns were run on SDS gel electrophoresis, and the results are shown on Figure 4.

Method 2:

The IB were sonicated repeatedly in 6 M urea, 0.05 M tris as pH 7.6. After centrifugation to get rid of the insoluble material, the sample was applied onto the Q-Sepharose and S-Sepharose columns as in Method 1.

Method 3:

Harvested cells of overexpressed *E coli* were suspended in lysis buffer (50 mM tris, 50 mM EDTA, 8% sucrose, 5% triton X-100, 0.1 mM Phenylmethyl-sulfonyl Fluoride (PMSF), and 0.5 mM DTT (Dithiothreitol). Lysozyme was added (0.5 mg/ml). It was then sonicated and centrifuged. The pellet was then washed 2-3 times with the above

mentioned triton X-100 buffer, sonicated and centrifuged. The process was repeated twice with the tris buffer without the triton X-100. The pellet was then dissolved into 6 M urea, 0.1 M NaCl, 50 mM tris, 1 mM EDTA, 0.1 mM PMSF, 0.5 M DTT at pH 8 with sonication. After centrifugation the sample was then applied onto first Q-Sepharose and the run through peak from the Q-Sepharose to S-Sepharose. The fraction from the S-Sepharose with Rop-Tat was dialyzed against o.1 % trifluoroacetic acid (TFA). The sample was then lyophilized to dryness, redissolved in 0.1% TFA, and applied onto a reverse phase HPLC C-18 column (Vydac 218TP54). The Rop-Tat was eluted with a gradient of 0.1% TFA to a 50% acetronitrile (in 0.1% TFA). The results are shown on Figure 5.

III. Conclusion

The first half of the reporting period was devoted to purifying Tat after cyanogen bromide cleavage of the fusion protein, Rop-Tat. After the last stage of purification by HPLC, purity of the sample is about 90%. The second half of the reporting period was used to purify Rop-Tat. The elution pattern from the S-Speharose (Figure 3) is slightly different depending on the method of sample preparation. There may exist at least two kinds of Rop-Tat. One is a monomer since both reducing and non-reducing SDS gel electrophoreses have the same protein band (gel result not shown). Another variety of Rop-Tat is a monomer under reducing conditions, but this monomer disappears under non-reducing with the appearance of a large band barely entering the separating gel from the stacking gel in the SDS electrophoresis. Further work is needed to clarify this observation, the results to be reported next quarter. We are trying to develop a method to purify Rop-

Tat without using HPLC since it is not only tedious to use HPLC, but the yield also becomes considerably lower. Method 3 gives us the best yields, even before using HPLC, and the purity approaches 90%. We are ready at this stage to refold the protein.

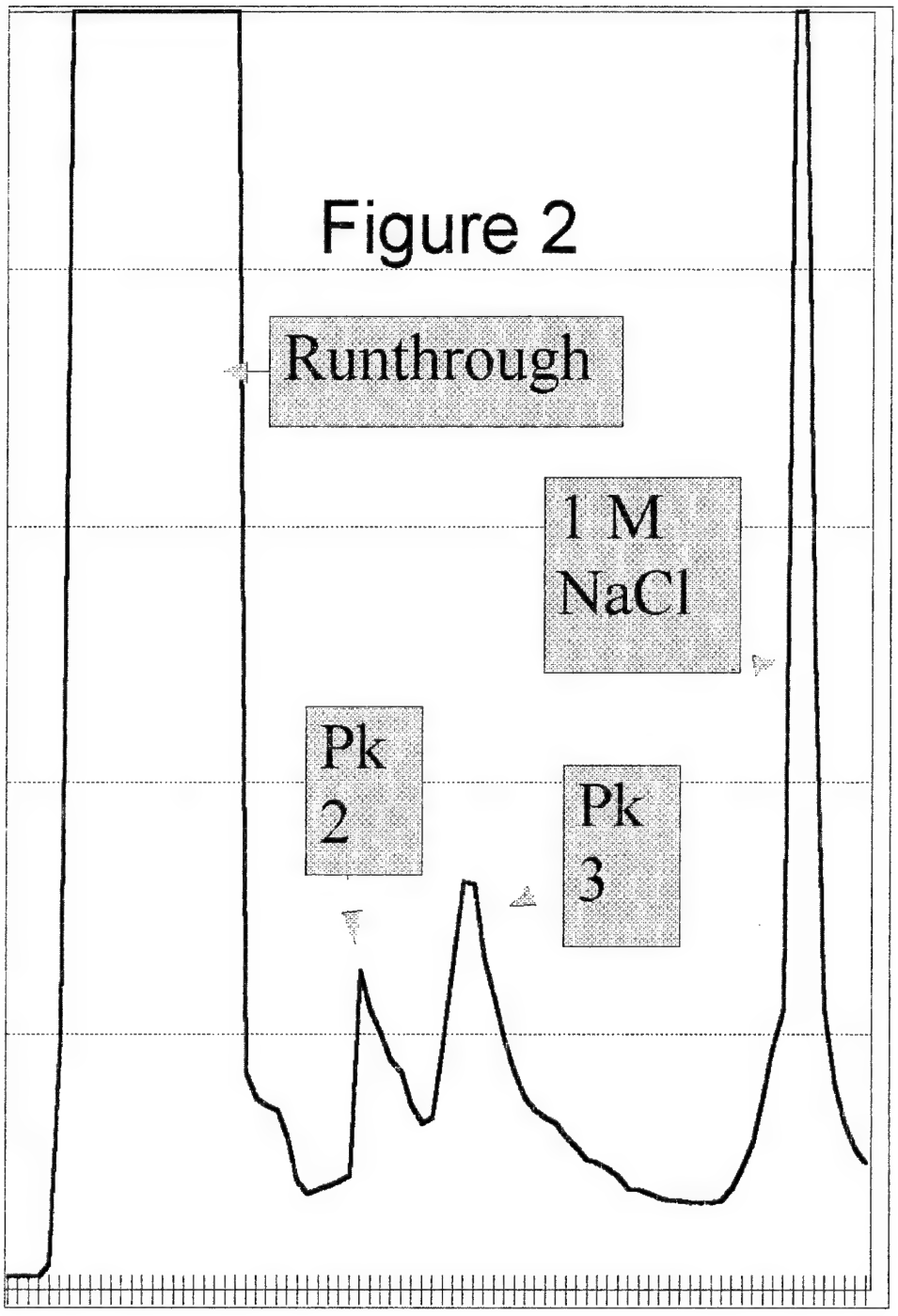

Figure 2. Q-Sepharose column chromatography (2.5 x 12 cm).
The column was equilibrated with 6 M urea, 0.05 M tris at pH 7.7.
After the run-through peak (Peak 1), the column was equilibrated
with a gradient of 0.1 M to 0.5 M NaCl in the above buffer. After
Peak 3, the column was eluted with 1 M NaCl in the urea, tris buffer.

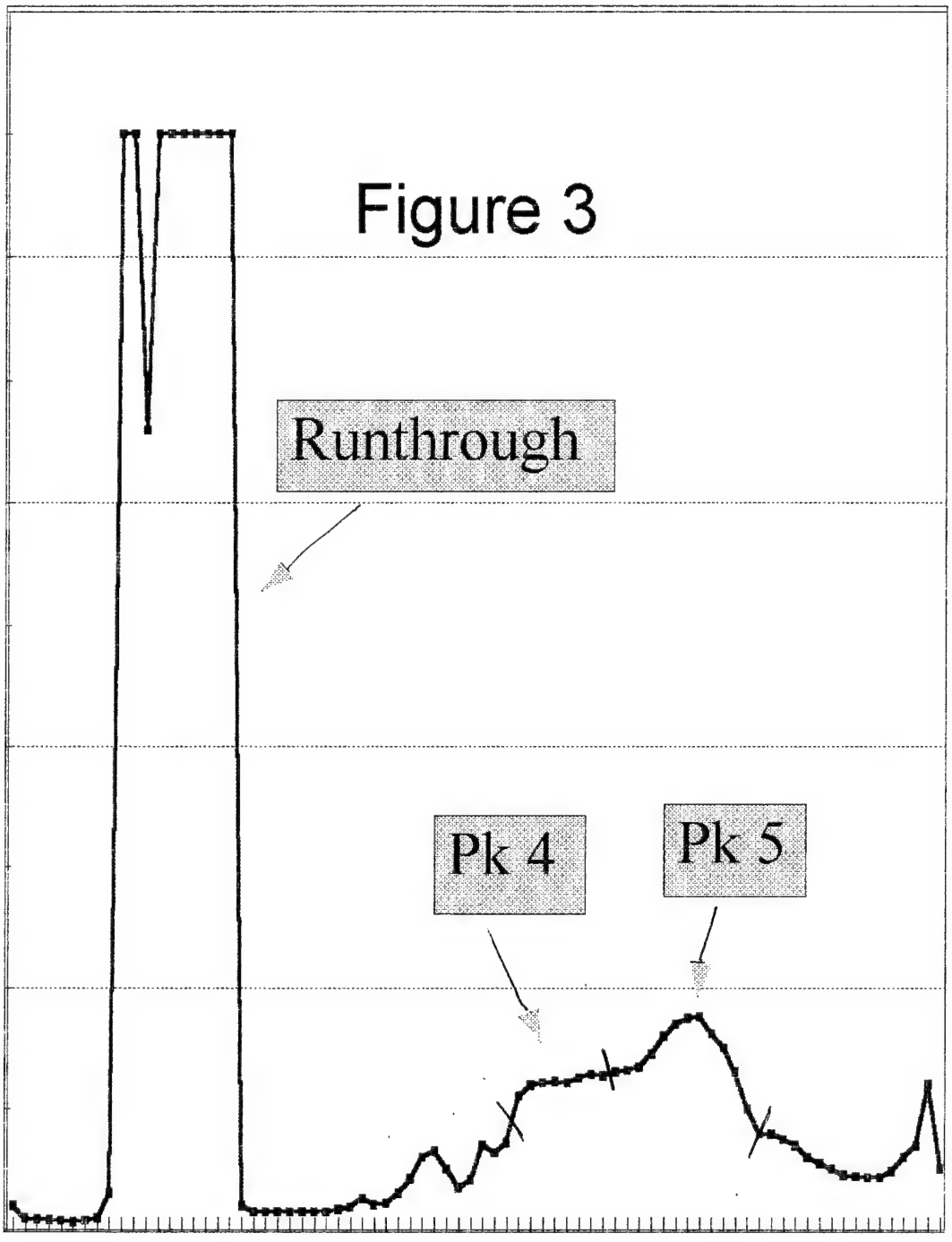

Figure 3. S-Sepharose chromatography of run-through fraction of Q-Sepharose (see Figure 2). The sample was loaded onto a column (2.5 x 12 cm). After the run-through peak, the column was eluted with a gradient of 0.1 M to 0.5 M NaCl in 6 M urea, 0.05 M sodium acetate at pH 5. The last peak (Peak 5) comes out near the end of the gradient.

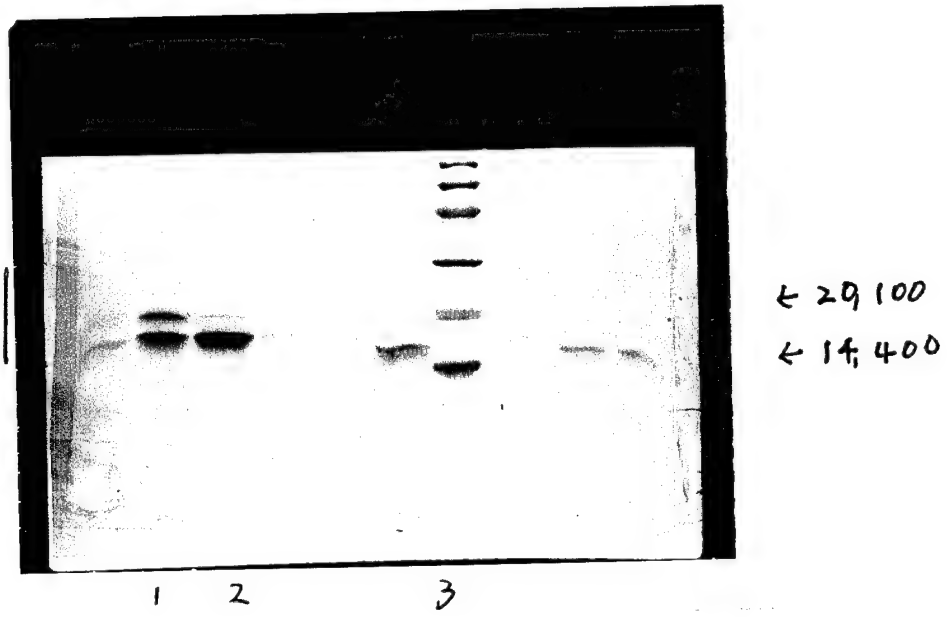

Figure 1 SDS-Gel Eletrophoresis

SDS-gel Electrophoresis in 15% acrylamide gel.

All samples are in reduced state.

Lanes 1 & 2 . Samples from HPLC

Lane 3. Molecular weight standard

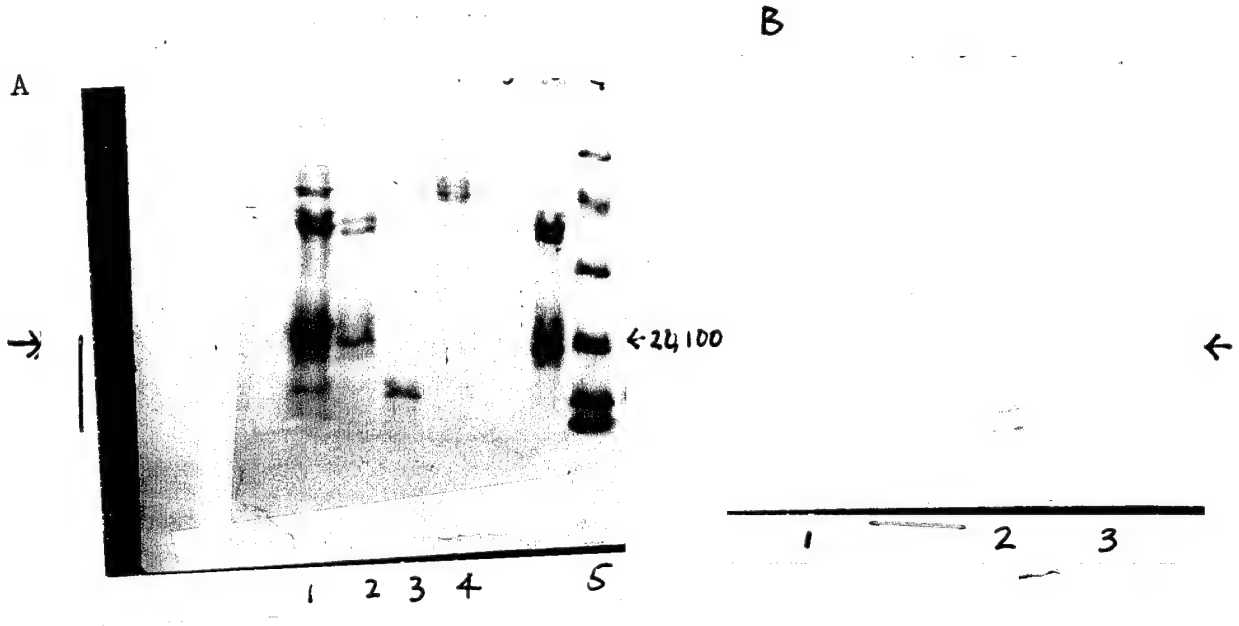

Figure 4. SDS-gel Electrophoresis of fractions from S & Q Sepharose of Figures 3 and 4. All samples are in reduced state.

Panel A - Lane 1, sample before Q-Sepharose (Figure 3)
Lane 2, Pk 1, Q-Sepharose
Lane 3 and Lane 4, Pk 2 & Pk 3 of Q-Sepharose respectively
Lane 5 Molecular weight standard

Panel B - Lane 1, Pk 4,S-Sepharose Figure 4. Lane 3, Pk 5 of S-Sepharose

The arrow indicates the position of Rop-Tat

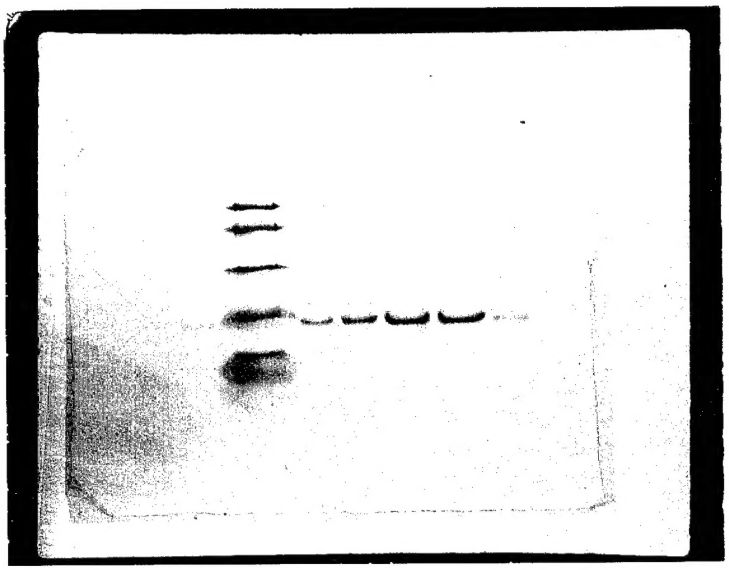

Figure 5. Sodium dodecyl sulfate (SDS) -polyacrylamide gel electrophoresis of samples from Method 3. Lanes 4,5, 6, 7 are fractions from HPLC . Lane m is molecular weight standards of 67,000, 43000, 30000,20100,14400,6000.

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APPENDIX

SUBCONTRACT OF CONTRACT DAMD 17-93-C-3122

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Johns Hopkins University

INTRODUCTION

During the past year, while studying HIV Tat regulated transactivation in Cos cells and in HeLa cell extract, we have concentrated our efforts on searching for compounds that can inhibit this step of the HIV life cycle. By inhibiting the HIV proviral expression in human cells, the drug holds the potential to suppress HIV replication: An alternative approach for studying AIDS control.

We have made a major breakthrough in research toward this goal. With dual support from NIH and from DAMD, we have isolated several anti-HIV lignans from a medicinal plant, *Larrea tridentata*. The structures of these lignans have been determined by GC/MS and NMR, and the biological activities of these anti-HIV lignans have also been analyzed using HIV-infected peripheral blood mononuclear cells and cell-free transcriptional systems. Three manuscripts have been submitted for publication. Two have already been accepted and the third is currently being reviewed. These manuscripts will be sent to the General Research Contracts Branch, Department of the Army within four weeks, after minor revisions.

RATIONAL OF THE PROJECT, PROGRESS MADE AND FUTURE PLANS:

Although all known HIV proteases and reverse transcriptase inhibitors are quite effective in eliminating wild type viruses, they have recently been found to be ineffectual in destroying a group of rapidly growing HIV mutants in infected patients. AIDS patients have shown no significant long term clinical improvement following treatments with these aforementioned drugs. New drug development aimed at wiping out these resistant HIVs thus becomes rather critical for AIDS control.

An alternative approach has been initiated recently to investigate whether the life cycle of all HIV variants can be interrupted at an early stage by inhibiting proviral gene expression. In other words, whether HIV-1 replication can be suppressed by HIV specific transcription inhibitors. Toward this goal, many medicinal plants have been screened and leaf extracts of creosote bush *Larrea tridentata* have been found to

possess such anti-HIV activity. Subsequently, five active compounds (FB_1-FB_5), each containing three phenolic hydroxyls including two in a catechol unit, have been isolated from this plant by assay-guided countercurrent chromatography (CCC). Their structures were determined by GC/MS and NMR. These compounds are derivatives of a plant lignan, 1,4-bis-(3,4-dihydroxyphenyl)-2,3-dimethyl butane (nordihydroquairetic acid, NDGA), an antioxidant commonly used to inhibit rancidity in the fats of food products. Among the five isolated pure lignans, FB_1 (4'-O-methyl NDGA, mol. wt. 316), FB_2 (3'-O-methyl NDGA, mol. wt. 316) and FB_4 (3'-dehydroxy NDGA, mol. wt. 286) are bicyclic lignans. FB_3 (3'-demethyl-oxy-8-O-demethylisoguaiacin, mol. wt. 284) and FB_5 (3'demethyoxy-6-O-demethylisoguaiacin, mol. wt. 284) are isomeric tricyclic lignans. The biological activity of Mal.4 (FB_2, 3'-O-methyl NDGA) has been studied extensively. Mal.4 was found to inhibit HIV Tat-regulated transactivation *in vivo*, induce protection of lymphoblastoid CEM-SS cells from HIV (strain III_B) killing and suppress the replication of five HIV-1 strains (WM, MN, VS, JR-CSF and III_B) in mitogen-stimulated peripheral blood mononuclear cells ($PBMC^+$), all in a dose-dependent manner. Mal.4 (but not NDGA) inhibits both basal transcription and Tat-regulated transactivation *in vitro* by directly and selectively interfering with the binding of Sp1 to Sp1 sites in the HIV LTR. Our immediate goals [05/24/95 - 05/23/96] are: (1) To study the mechanism underlying the drug effect on Sp1 regulated transcription: whether these lignans (FB_1-FB_5) act either as general transcriptional inhibitors by binding to SP1 protein or Sp1 sites in the promoter, or act differently by inducing DNA structural distortion in the vicinity of Sp1 sites, thus indirectly interfering with the Sp1/Sp1 site interaction required for basal transcription and transactivation. (2) To study the anti-HIV activities of FB_1, FB_3, FB_4 and FB_5, and to relate the structural requirement of the lignans in suppressing HIV-1 replication in infected PBMC as previously tested for FB_2 (Mal.4). (3) To conduct pharmaco-kinetic studies in infected SCID/Hu mice comparing the effectiveness and level of toxicities of these lignans. Our long term goals [a request for future funding is in preparation] are aimed at designing and synthesizing specific compounds for treatment of AIDS patients. These natural anti-HIV lignans have been chosen as chemotypes for the drug design, since chemicals similar to FBs have been detected in tissues of healthy humans. They may thus be safe to be used clinically.